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Antigenic and immunogenic properties of recombinants from *Salmonella typhimurium* and *Salmonella minnesota* rough mutants expressing in their lipopolysaccharide a genus-specific chlamydial epitope.
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reparation and structural analysis of oligosaccharide monophosphates obtained from the lipopolysaccharide of recombinant strains of *Salmonella minnesota* and *Escherichia coli* expressing the genus-specific epitope of *Chlamydia* lipopolysaccharide.
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Antigenic and Immunogenic Properties of Recombinants from *Salmonella typhimurium* and *Salmonella minnesota* Rough Mutants Expressing in Their Lipopolysaccharide a Genus-Specific Chlamydial Epitope

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Rough mutants from *Salmonella typhimurium* and *Salmonella minnesota* were transformed with a plasmid containing a 6.5-kilobase insert of DNA from *Chlamydia trachomatis* assumed to encode a glycoyltransferase. Transformation resulted in the expression of a genus-specific chlamydial epitope on the lipopolysaccharide (LPS) of the recombinant strains. Proteinase K-digested whole-cell lysates of the recombinants and of controls were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver staining or Western blot analysis. Two LPS populations were detected in the recombinants, the parent LPS and a faster-migrating component. The latter stained with monoclonal antibody against the genus-specific chlamydial epitope and was not seen in the controls. LPS was extracted and purified from recombinants of *S. minnesota* R595 and R4 and characterized by the passive hemolysis and passive hemolysis inhibition assays and by hydrolysis kinetics. Different antigenic determinants could be distinguished from each other by the passive hemolysis inhibition test with monospecific antigen-antibody reactions. Rabbits were immunized with heat-killed recombinant bacteria to study the immunogenic properties of the recombinants. In all animals, antibodies were raised against the parent core specificity and against the chlamydia-specific epitope. The data show that the recombinant bacteria are useful as immunogens to prepare polyclonal antisera against chlamydiae and that LPS isolated from them exhibits the same antigenic determinants as chlamydial LPS and may thus be used as a substitute for chlamydial LPS in serological assays.

Chlamydiae are pathogenic, obligatory intracellular parasites causing a variety of diseases in animals and humans (18, 29). Chlamydiae have a unique developmental cycle including metabolically active, noninfectious, multiplying reticulate bodies and metabolically inactive but infectious elementary bodies (27). Surface components of these particular bacteria have been assumed to participate in the early steps during infection (adhesion and penetration) and may be responsible for the inhibition of phagosome-lysosome fusion, a characteristic feature of the chlamydial infection (8, 10, 15).

At the same time, these surface components of chlamydiae also represent antigens among which genus-, species-, and subspecies-specific antigens have been found (12, 27). Little is known about the serological and biological properties of these antigens (1, 4-6, 9-14, 28) and even less is known about their chemistry (13, 14, 23). One of the major antigens is a heat-stable, genus-specific glycolipid antigen (13, 14) which has been proposed to be similar to the lipopolysaccharide (LPS) of gram-negative bacteria (4, 6, 9, 13, 22-24). We have shown definitely, by chemical means, that the glycolipids of *Chlamydia trachomatis* serotype L2 (23) and of *Chlamydia psittaci* (7) are in fact typical LPS, since they contained characteristic structural elements such as D-glucosamine, 3-deoxy-D-manno-2-octulosonic acid, phosphate, and 3-hydroxy long-chain fatty acids, compo-

nents which have been detected in all LPS investigated so far (25). Further evidence for the similarity between chlamydial and enterobacterial LPS was obtained from serological investigations showing a cross-reaction of chlamydial with enterobacterial LPS of Re chemotype (4, 9, 24) and with LPS from *Acinetobacter calcoaceticus* (2, 24).

Caldwell and Hitchcock (9) have reported on a monoclonal antibody which recognizes a genus-specific epitope on the chlamydial LPS which did not react with Re LPS in Western blot analysis. Thus, the chlamydial LPS expresses at least two antigenic determinants, one of which is chlamydia specific, and the other being similar to the Re antigenic determinant. We have confirmed these results by using polyclonal antisera against chlamydial and Re-type LPS in a passive hemolysis assay, and moreover, we have demonstrated by absorption experiments the presence of two antibody specificities in polyclonal antisera against *C. trachomatis* (6). One of these antibodies reacted with both chlamydial and Re LPS, and the other was chlamydia specific. In addition, we have shown that chlamydial LPS contains the lipid A antigenic determinant which, as in other bacteria, is cryptic in LPS and exposed after acid hydrolysis (6).

Recently, Nano and Caldwell (21) reported the molecular cloning of a DNA fragment of *C. trachomatis* in *Escherichia coli* by using the pUC8 plasmid as a vector. Transformants which contained the recombinant plasmid expressed, in addition to the parental LPS, a second, modified LPS

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population which had a similar size as LPS of enterobacterial Re mutants and expressed the aforementioned chlamydia-specific LPS epitope as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining and Western blot analysis. The authors hypothesized that the cloned DNA fragment encoded a glycosyl transferase which added to the biosynthetically growing LPS a sugar which determined the chlamydia-specific epitope and at the same time blocked further biosynthesis of the parent LPS.

These recombinants combine ease of growth with the expression of the chlamydia-specific epitope and are thus a convenient source for the preparation of large quantities of LPS harboring the genus-specific chlamydial epitope. It will be shown below that this LPS can be used as a substitute for chlamydial LPS in serological assays.

Here we report on the antigenic and immunogenic properties of such recombinants and the LPS isolated from them.

MATERIALS AND METHODS

Bacteria and bacterial LPS. *Salmonella typhimurium* chemotypes Ra (strain SL3749), Rb₁ (strain SL733), Rb₂ (strain SL3750), Rc (strain SGCS), Rd₁ (strain SL3769), Rd₂ (strain SL3789), and Re (strain SA1377) and *Salmonella minnesota* chemotypes Re (strain R595) and Rd₂ (strain R4) were transformed with plasmid pFEN207 which contains a 6.5-kilobase insert of *Sau*3A-digested DNA from *C. trachomatis* (21). Controls (without the insert) were cultivated in parallel. *S. typhimurium* strains were grown on Loeb agar with ampicillin (30 µg/ml). Whole-cell lysates were prepared and digested with proteinase K as described previously (9). *S. minnesota* bacteria were grown on a large scale in a 14-liter fermentor in the presence of ampicillin at a concentration of 80 µg/ml (R4) or 60 µg/ml (R595). From these two strains LPS was extracted by the phenol-chloroform-petroleum ether method (17), purified by repeated ultracentrifugation, converted to the uniform triethylammonium salt after electrodialysis (16), and designated *S. minnesota* R595-207 and *S. minnesota* R4-207, respectively. LPS of *C. psittaci* (ewe abortion strain [26]) was prepared from purified yolk sac-grown elementary bodies as described previously (23). LPS from *C. trachomatis* serotype L2 (strain 434Bu [23]), *S. minnesota* Re (strain R595 [5]), and bisphosphorylated *E. coli* lipid A (5) were those described in the respective references. De-O-acylated LPS (LPS-OH) was prepared with sodium methylate (0.5 M, 37°C for 16 h), precipitated and washed with ethanol, and dissolved in water.

Antibodies. Monoclonal antibody against chlamydiae was the one described by Caldwell and Hitchcock (9). It is an immunoglobulin G3 antibody recognizing a genus-specific epitope on chlamydial LPS and is designated L2I-6 (9). Polyclonal antisera against *C. psittaci* and recombinant *S. minnesota* strains were prepared in rabbits as follows. New Zealand White rabbits, pretested for the absence of antibodies against the chlamydial group-specific antigen or the rough LPS under investigation, were injected intravenously with Formalin-killed *C. psittaci* elementary bodies or heat-killed (100°C for 2 h) bacteria. The animals received 50, 100, 100, and 200 µg on days 0, 4, 7, and 11, respectively, and were bled on day 16. Polyclonal antisera against *S. minnesota* R595 and R4 LPS and against bisphosphorylated lipid A were prepared as already reported (5, 20). Antisera were

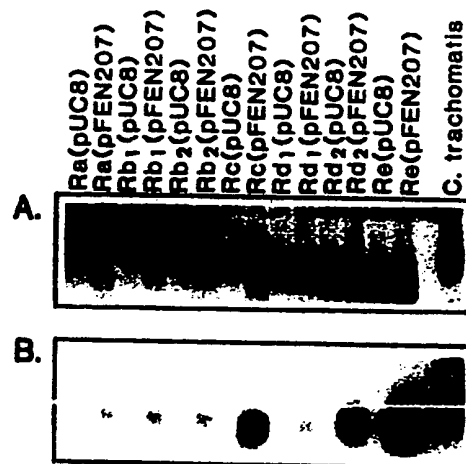


FIG. 1. SDS-PAGE of whole-cell lysates from *S. typhimurium* rough mutants transformed with plasmid pFEN207 containing a 6.5-kilobase insert from DNA of *C. trachomatis*. Controls contained the pUC8 plasmid without the chlamydial insert. LPS was visualized with alkaline silver nitrate (A) or, after Western blot transfer, with monoclonal antibody L2I-6 against the chlamydia-specific epitope followed by incubation with ¹²⁵I-labeled protein A and autoradiography (B).

absorbed with sheep erythrocytes (SRBC) and stored at -20°C.

Serological methods. Antibodies were determined in microtiter plates by the passive hemolysis test (5) with guinea pig complement, pretested for the absence of chlamydial antibodies, and absorbed with SRBC. Inhibition studies and absorption experiments were performed as described previously (6). Evaluations of hydrolysis kinetics of LPS were carried out in acetate buffer (0.1 M, pH 4.4) at 100°C for various times to study the behavior of different antigenic determinants upon acid hydrolysis. The hydrolyzed samples (0.5 mg each at a concentration of 1 mg/ml) were neutralized with triethylamine and used as inhibitors in the passive hemolysis inhibition assay.

SDS-PAGE and Western blot transfer. SDS-PAGE of LPS was done on proteinase K-digested whole-cell lysates (9) by the method of Laemmli (19) with a 16% gel. Gels were stained with silver nitrate by the method of Tsai and Frash (30). For Western blot analysis, the gels were transferred electrophoretically to nitrocellulose and developed with monoclonal antibody L2I-6 followed by incubation with ¹²⁵I-labeled protein A as described previously (1, 9).

RESULTS

SDS-PAGE pattern of LPS from *S. typhimurium* recombinants. Whole-cell lysates of *S. typhimurium* rough mutants being transformed with plasmid pFEN207 were digested with proteinase K and subjected to SDS-PAGE. The same strains containing the pUC8 plasmid without the chlamydial insert were used as a control. LPS was visualized with silver stain or, after Western blot transfer, with monoclonal antibody L2I-6. The results are shown in Fig. 1. One major band was stained with alkaline silver nitrate (Fig. 1A), which represents the parent LPS since it was similarly present in samples from strains with or without the recombinant plasmid. The various chemotypes exhibited a slightly different migration pattern owing to the different sizes of the core

TABLE 1. Hemolytic antibody titers in rabbits after immunization with recombinants of *S. minnesota* R595 and R4

Immunizing strain and animal no.	Hemolytic antibody titer against SRBC coated with LPS-OH from ^a :				
	<i>S. minnesota</i> R595	<i>S. minnesota</i> R4	<i>S. minnesota</i> R595-207	<i>S. minnesota</i> R4-207	<i>C. psittaci</i>
<i>S. minnesota</i> R595-207					
139	512	16	1,024	2,048	8,192
140	512	64	2,048	2,048	8,192
143	512	<4	512	512	2,048
144	2,048	8	2,048	2,048	16,384
<i>S. minnesota</i> R4-207					
145	32	2,048	1,024	2,048	1,024
146	64	2,048	512	2,048	1,024

^a SRBC (200 μ l of packed cells) were coated with 80 μ g of the respective LPS-OH.

oligosaccharide. The LPS from *C. trachomatis* had a size comparable to that of the Rc LPS. In addition, a second, faster-migrating band was observed in samples from those strains containing the pFEN207 plasmid, the intensity of which varied within the different strains. This band stained with monoclonal antibody against the chlamydia-specific LPS epitope (Fig. 1B). Therefore, it represents the LPS population expressing the chlamydia-specific epitope.

Immunization of rabbits with recombinant strains of *S. minnesota* R595-207 and R4-207. Rabbits were immunized with heat-killed bacteria according to a short-term immunization protocol resulting in immunoglobulin M-rich immune sera which were tested by the passive hemolysis assay. The results are shown in Table 1. In all animals, antibodies were raised against LPS from the parent and recombinant strains with titers of up to 2,048. They also reacted with LPS from *C. psittaci*, and the titers were higher in animals immunized with *S. minnesota* R595-207 than in those immunized with R4-207. Thus, immunization with the recombinants resulted in the formation of antibodies against the parent core specificity (Re and Rd₂, respectively) and against the chlamydia-specific epitope.

Antigenic properties of LPS isolated from recombinant strains. LPS was isolated from *S. minnesota* R595-207 and R4-207, and the antigenic properties were studied by the passive hemolysis assay (coating efficiency for SRBC), by the passive hemolysis inhibition assay (ability to inhibit different antibody specificities), and by hydrolysis kinetics

(chemical stability of various antigenic determinants). Table 2 shows the hemolytic titers obtained with polyclonal antisera and monoclonal antibody L2I-6 against SRBC sensitized with increasing amounts of LPS-OH from recombinants. Monoclonal antibody against the chlamydia-specific epitope and rabbit polyclonal antisera against *C. psittaci*, R595, and R4 reacted with the corresponding antigens, indicating that the LPS from recombinants contained the antigenic determinants from the parent LPS and the chlamydia-specific epitope. To further support this assumption, we performed inhibition experiments by using hemolytic antigen-antibody systems which were specific for the chlamydial epitope (*C. psittaci* LPS-OH-monoclonal antibody L2I-6), the Re specificity (R595 LPS-OH-anti-R595), or the Rd₂ specificity (R4 LPS-OH-anti-R4). In addition, the antigen-antibody system of R4-207 LPS-OH-anti-*C. psittaci* (from which the Re antibodies had been absorbed with R595 LPS-OH) was included. The latter system should allow us to answer the question as to whether the pure chlamydia-specific system (authentic chlamydial LPS and monoclonal antibody) could be substituted with polyclonal antiserum and LPS from recombinants. The result is shown in Table 3. The R4-207 LPS inhibited the R4- and R595-specific systems with 63 ng, indicating that the Re and Rd₂ determinants were expressed in this preparation. However, a by far more effective inhibition was obtained in the chlamydia-specific system, yielding similar inhibition values of 4 and 8 ng, respectively. A comparable result was obtained with the

TABLE 2. Hemolytic antibody titers of polyclonal rabbit antisera and mouse monoclonal antibody L2I-6 against SRBC coated with various amounts of homologous and heterologous LPS-OH

Sensitizing antigen (LPS-OH)	Amt of antigen (μ g/200 μ l of SRBC)	Hemolytic antibody titer obtained with:					
		Monoclonal L2I-6	Anti- <i>C. psittaci</i> ^a	Anti- <i>S. minnesota</i> R595	Anti- <i>S. minnesota</i> R595-207 ^b	Anti- <i>S. minnesota</i> R4	Anti- <i>S. minnesota</i> R4-207 ^c
<i>S. minnesota</i> R595-207	4	20	160	80	320	NT ^d	NT
	8	40	320	160	640	NT	NT
	40	160	640	640	1,280	NT	NT
	80	160	1,280	1,280	1,280	NT	NT
	200	160	1,280	1,280	1,280	NT	NT
<i>S. minnesota</i> R4-207	4	<10	<10	NT	NT	20	20
	8	20	10	NT	NT	1,280	40
	40	160	640	NT	NT	5,120	640
	80	160	1,280	NT	NT	5,120	2,560
	200	160	1,280	NT	NT	5,120	2,560

^a The antiserum was absorbed with R595 LPS-OH.

^b Animal no. 139 from Table 1.

^c Animal no. 145 from Table 1.

^d NT, Not tested.

TABLE 3. Passive hemolysis inhibition test of LPS from recombinants of *S. minnesota* R595 and R4

Inhibitor	Inhibition value (ng) obtained in the indicated hemolytic antigen-antibody system ^a			
	<i>C. psittaci</i> LPS-OH-L21-6	R4-207 LPS-OH- anti- <i>C. psittaci</i> ^b	R595 LPS-OH- anti-R595	R4 LPS-OH- anti-R4
R4-207 LPS	8	4	63	63
R595-207 LPS	4	2	32	>1,000

^a Three hemolytic units of antibody were used.^b The antiserum was absorbed with R595 LPS-OH.

R595-207 LPS which inhibited both chlamydia-specific systems with amounts of 4 and 2 ng, respectively. The Re-specific antigen-antibody reaction was inhibited with 32 ng, and as expected, the Rd₂-specific system was not inhibited at all (inhibition value of >1,000 ng).

Stability of various antigenic determinants in LPS from *S. minnesota* R4-207 toward acid. Since it is known from many LPS that the polysaccharide determinants are split off during acid hydrolysis, evaluations of hydrolysis kinetics were performed under controlled pH. The hydrolyzed samples were tested by the passive hemolysis inhibition test, using the same assays as before. In addition, bisphosphorylated *E. coli* lipid A and anti-lipid A antiserum were used to detect the lipid A antigenic determinant which is cryptic in all LPS investigated so far. The Re-, Rd₂-, and chlamydia-specific determinants were destroyed during hydrolysis (Fig. 2). Similar kinetics were observed for the Rd₂ and the chlamydial determinants, whereas the Re specificity seemed to be destroyed faster as indicated by inhibition values of 500 and >1,000 ng after 15 and 30 min, respectively. Lipid A antigenicity was not detected in the unhydrolyzed LPS but was exposed during hydrolysis, reaching a maximum inhibition value of 32 ng after 60 min.

DISCUSSION

The genus-specific antigen of chlamydiae is an LPS which is structurally similar to enterobacterial LPS (7, 23). It carries at least two epitopes, one of which cross-reacts with enterobacterial LPS of the Re type, and the other being chlamydia specific (6, 9, 24). The molecular cloning of a chlamydial DNA fragment in *E. coli* has been reported (21), resulting in the expression of the chlamydia-specific epitope on the LPS of these recombinants. Now, we transformed rough mutants of *S. typhimurium* and *S. minnesota* to see whether the chlamydial epitope can be also expressed in rough mutants, which have been well characterized (20). We found that all chemotypes of *S. typhimurium* (Ra through Re) could be transformed with the recombinant plasmid pFEN207 (21). SDS-PAGE analysis followed by silver stain or Western blot transfer indicated that two LPS populations were present, one of which represented the parent rough form LPS, and the other being smaller in size (Fig. 1). Using monoclonal antibody against the genus-specific epitope of chlamydiae (9), it was shown that this epitope was only expressed on the faster-migrating LPS and not on the parent LPS. This is in accordance with the previously postulated assumption that the cloned DNA fragment encodes for a glycosyltransferase modifying the biosynthetically growing LPS chain in such a way that the biosynthesis of the parent LPS is blocked (21). This results in a truncated LPS molecule expressing at the same time the chlamydia-specific epitope. The extent of expression varied, however, within the different strains, an observation for which no explanation can be given now.

Two similarly prepared recombinants of *S. minnesota* rough mutants R595 and R4 were investigated in more detail. LPS was extracted from these recombinants, and the antigenic properties were investigated. In addition, polyclonal antisera were raised in rabbits with heat-killed recombinants as immunogens. These antisera contained antibodies against the parent core specificity, i.e., the Re and Rd₂ antigenic determinants, respectively, and against the chlamydial LPS epitope, indicating that both determinants are immunogenic for rabbits. It was demonstrated by inhibition experiments with isolated LPS and monospecific antigen-antibody systems that these antigenic determinants could be differentiated from each other. Hydrolysis kinetics indicated that the determinants were all destroyed by mild acid hydrolysis. This suggests that the chlamydial epitope generated through the action of the cloned transferase is related to a not yet identified sugar moiety which itself either exhibits an acid-labile linkage or is linked to one of the 2-keto-3-deoxy octulosonic acid residues in the inner core region (3, 4).

LPS from recombinants will be available in larger quantities to allow structural investigations; thus, we expect that the chlamydial epitope will be defined chemically on a molecular level in the near future.

The recombinants are already a useful tool. They are a convenient source for the genus-specific antigen to be used in serological studies. Our data show that the hemolytic titers of monoclonal antibody and polyclonal antisera can be determined with the same accuracy when using either chlamydial or recombinant LPS as a test antigen. Moreover, in inhibition experiments, the pure chlamydia-specific antigen-

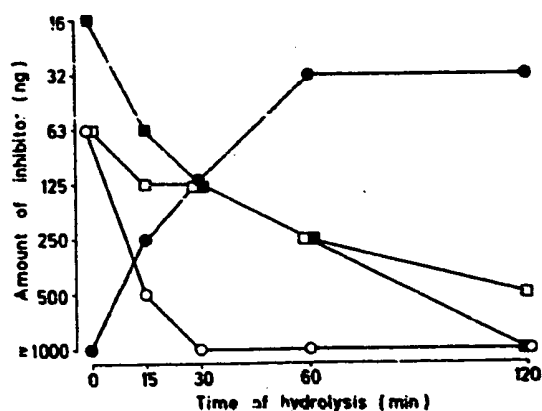


FIG. 2. Hydrolysis kinetics of recombinant LPS in acetate buffer (0.1 M; pH 4.4; 100°C). Samples of R4-207 LPS were hydrolyzed for various times (indicated on the abscissa) and tested in the passive hemolysis inhibition assay with the following antigen-antibody systems: *C. psittaci* LPS-OH-monoclonal antibody L21-6 (■), *S. minnesota* R4 LPS-OH-anti-R4 (□), *S. minnesota* R595 LPS-OH-anti-R595 (○), and *E. coli* lipid A-anti-lipid A (●).

antibody system (chlamydial LPS and monoclonal antibody) can be substituted with LPS from recombinants and polyclonal antiserum (Table 3). The LPS from recombinants of Re mutants harbors the same antigenic determinants as chlamydial LPS: the Re specificity and the chlamydia-specific epitope. This cross-reactivity between chlamydial and Re-type LPS makes the interpretation of antibody titers difficult. Those antibodies reacting with chlamydial but not with Re LPS are clearly chlamydia specific; however, those reacting with both LPS may have been elicited by contact with either Re mutant bacteria or chlamydiae.

The chemical structure of the LPS from chlamydiae and recombinants is presently being investigated in our laboratory. It is expected that these studies will allow us to separate these two antigenic determinants of chlamydial LPS chemically.

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Chlamydia Trachomatis DNA Encodes Homologues of Salmonella MVI and C. Pneumoniae 76KD Proteins

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CHLAMYDIA TRACHOMATIS DNA ENCODES HOMOLOGUES OF
SALMONELLA *mvi* AND C. PNEUMONIAE 76kD PROTEINS

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A segment of *Chlamydia trachomatis* genomic DNA containing three open reading frames (ORFs 1-3) that may encode membrane associated proteins was characterised.

The first ORF codes for a hydrophobic protein of 540 amino acids. We found 20% identity and 43% similarity between the derived amino acid sequences of ORF1 and the *mvi* gene from *Salmonella typhimurium*. Hydropathy analysis of both the amino acid sequences produced similar plots. There are 14 trans-membrane segments and computational prediction using PSORT algorithms suggest that ORF1 encodes an inner membrane protein.

The *mvi* gene product in *S. typhimurium* may be a virulence factor (Sanderson *et al.* 1995). Insertional inactivation of *mvi* attenuated *S. typhimurium* in a "burnt mouse" model (Carsiotis *et al.* 1989). A similar gene is also present in another Gram-negative pathogen, *Haemophilus influenzae*.

Immediately downstream from the *mvi*-homologue was a 632 bp open reading frame (ORF2) encoding a protein of 182 amino acid residues. The N-terminal 21 residues appear to have characteristics of a signal peptide sequence, suggesting this predicted protein may be transported out of the cytoplasm. Database searches have not revealed any significant matches to known sequences.

Another open reading frame was found further downstream (ORF3). We have sequenced approximately 800bp. Analysis showed that the translated sequence of ORF3 is homologous to the *C. pneumoniae* 76kD protein which has species specific epitopes (McGosa *et al.* 1994).

At the nucleic acid level, the similarity between ORF3 sequence and the corresponding region of the gene for the 76kD protein from *C. pneumoniae* is 56%. At the amino acid level, they share 58% identity and 73% similarity in the same region.

Southern hybridisation to the genomic DNA from *C. trachomatis* serovars B and L2 revealed that the organisation of these three ORFs are similar in the two biovars of *C. trachomatis*. No homologous sequences were detected in *C. psittaci* genomic DNA.

Our results suggest that the observed species-specific immune response to the *C. pneumoniae* 76kD protein (McGosa *et al.* 1994) is due to antigenic diversity.

Northern blots of RNA from *C. trachomatis* identified the transcripts corresponding to ORF1 and ORF3.

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